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RESEARCH PAPER

Mapping of quantitative trait loci associated with chilling tolerance in maize (*Zea mays* L.) seedlings grown under field conditions

Choosak Jompuk, Yvan Fracheboud, Peter Stamp and Jörg Leipner*

Institute of Plant Sciences, Swiss Federal Institute of Technology Zurich, Universitätstrasse 2, CH-8092 Zürich, Switzerland

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Abstract

The effect of low growth temperature on morpho-physiological traits of maize was investigated by the means of a QTL analysis in a segregating $F_{2:3}$ population grown under field conditions in Switzerland. Chlorophyll fluorescence parameters, leaf greenness, leaf area, shoot dry weight, and shoot nitrogen content were investigated at the seedling stage for two years. Maize was sown on two dates in each year; thus, plants sown early were exposed to low temperature, whereas those sown later developed under more favourable conditions. The main QTLs involved in the functioning of the photosynthetic apparatus at low temperature were stable across the cold environments and were also identified under controlled conditions with sub-optimal temperature in a previous study. Based on the QTL analysis, relationships between chlorophyll fluorescence parameters and leaf greenness were moderate. This indicates that the extent and functioning of the photosynthetic machinery may be under different genetic control. The functioning of the photosynthetic apparatus in plants developed at low temperature in the field did not noticeably affect biomass accumulation; since there were no co-locations between QTLs for leaf area and shoot dry weight, biomass accumulation did not seem to be carbon-limited at the seedling stage under cool conditions in the field.

Key words: Chilling tolerance, chlorophyll fluorescence, field environment, maize, photosynthesis, QTL, seedling vigour.

Introduction

During the latter part of the last century, the cultivation of maize (*Zea mays* L.), which originated in the subtropics, has been extended to higher latitudes. Adaptation to growth conditions, as in northern Europe, has been partially successful due to breeding for early maturing maize plants and, therefore, reaching a compromise between the risk of yield loss and an acceptable level of yield (Stamp, 1986). Nevertheless, the chilling-sensitive nature of maize makes early plant establishment in spring difficult under cool environmental conditions.

Among the various effects of low temperature on the physiology of maize, that on the photosynthetic apparatus is considered to be especially important (Baker *et al.*, 1994). In particular, the combination of high light intensity and low temperature, as occurs frequently during the early growing season in temperate regions, can cause photoinhibition of photosynthesis (Farage and Long, 1987). Leaves of maize, which develop under such conditions, are characterized by a lower photosynthetic capacity, lower quantum efficiency of CO_2-fixation (ϕCO_2), and lower quantum efficiency of electron transfer at PSII (ϕ_{PSII}) than leaves which develop under more favourable conditions (Nie *et al.*, 1992; Fryer *et al.*, 1998; Leipner *et al.*, 1999). One reason for the lower photosynthetic performance might be the perturbation of chloroplast development, specifically, the limited ability of maize leaves to develop a functional photosynthetic apparatus at low temperature (Nie and Baker, 1991). Besides this, the susceptibility of the enzymes involved in the C_4-cycle, especially that of pyruvate orthophosphate dikinase, are discussed as being the cause of the chilling sensitivity of the photosynthetic machinery of C_4-plants, and in particular of maize (for

* To whom correspondence should be addressed. Fax: +41 1 632 1143. E-mail: joerg.leipner@ipw.agrl.ethz.ch

a review see Long, 1983). Moreover, ribulose *bis*phosphate carboxylase (Rubisco) activity is known to be reduced in maize seedlings at low temperature (Kingston-Smith *et al.*, 1997). Its activity has been found to be the limiting factor for photosynthesis at suboptimal temperature in another C_4-plant, *Muhlenbergia glomerata* (Kubien and Sage, 2004). Recently, the necessary intercellular partitioning of the antioxidative defences between the mesophyll and bundle sheath and probably also a potential disruption of the circadian regulation of certain photosynthetic enzymes are claimed to cause the chilling sensitivity of maize (Foyer *et al.*, 2002). Furthermore, the transport rate of assimilates between the mesophyll and the bundle sheath, as well as phloem loading, are considered to be affected by low temperature (Sowinski *et al.*, 1998, 2003). Whatever is the primary cause of the chilling-induced reduction of photosynthesis, its effect on seedling growth has only been studied by the comparison of a few genotypes with contrasting chilling tolerance (Verheul, 1992; Verheul *et al.*, 1995), which does not yet allow a final conclusion to be drawn concerning the importance of photosynthesis for growth under low temperature conditions.

For some years, the use of molecular markers have enabled the identification of quantitative trait loci (QTLs) involved in the expression of important agronomic traits (e.g. yield components) (Kraja and Dudley, 2000) or disease resistance (Moon *et al.*, 1999). Although the genetic dissection of drought tolerance in maize has been studied extensively (for a review see Ribaut *et al.*, 2004), less is known about the genetic basis of chilling tolerance: research under controlled conditions has only recently been undertaken in this laboratory (Fracheboud *et al.*, 2002, 2004). Not only is QTL analysis important in breeding programmes, it is also a powerful tool for studying the relationships between complex physiological traits (for a review see Prioul *et al.*, 1997). Although complex processes like biomass accumulation are controlled by a large number of genetic factors, only a few of these processes are supposed to be limiting factors. Moreover, the importance of genetic factors for a certain trait might be considerably affected by environmental conditions, making the genetic dissection of physiological traits by means of QTL analysis a promising approach for stress physiologists.

Studies of chilling stress conducted under controlled conditions, however, often poorly reflect natural environmental conditions which are usually characterized by considerable fluctuations in temperature and light intensity. Hence, it is not surprising that growth of maize at an early stage of development under field conditions often shows little correlation with maize grown under controlled conditions (Revilla *et al.*, 1998). Therefore, the aim of the present study was to conduct a QTL analysis in order to understand the genetic and physiological mechanism(s) of chilling tolerance of maize seedlings under field conditions. To do this, a segregating population, which was studied previously under controlled conditions (Fracheboud *et al.*, 2004), was sown on two different dates under temperate climatic conditions in Switzerland. Thus, plants sown early were exposed to chilling conditions, whereas plants sown later developed under conditions more favourable for growth. The QTL analysis of morpho-physiological traits and the identification of potential candidate genes may help to understand the relationship between photosynthesis and growth in different environments as well as the genetic background of chilling tolerance in maize.

Materials and methods

Plant material

Maize (*Zea mays* L.) lines with contrasting chilling-tolerance of photosynthesis were obtained by divergent selection from a Swiss dent maize breeding population using chlorophyll fluorescence as the selection tool (Fracheboud *et al.*, 1999). Two lines in the S5 generation, namely ETH-DH7 (chilling-tolerant) and ETH-DL3 (chilling-sensitive) were used as parents to produce a segregating F_2 population. From 254 F_2 plants a genetic linkage map was constructed using simple sequence repeat markers (Fracheboud *et al.*, 2004). The F_2 plants were grown in the field and selfed, yielding 226 successful F_3 families. Of each F_3 family, 20 plants were grown in the field and intercrossed within families to produce the $F_{2:3}$ population used in the present QTL experiments.

Field experiments

In 2002, the $F_{2:3}$ families were sown on two dates: 26 April (early sowing) and 24 May (late sowing). In 2003, sowing was done on 14 April (early) and 15 May (late). The experimental unit was a single-row plot with 50 plants, 5 m long, and 0.75 m between the rows. All the experiments were over-planted by machine and later thinned to the final plant number. Trials of 226 $F_{2:3}$ lines and the two parental lines were conducted using an alpha (0,1) lattice design with 23 blocks per replication (Patterson and Williams, 1976) and two replications for each sowing date. Each replication was bordered by two rows of a mixture of the $F_{2:3}$ families. In each experimental unit, the first two plants were considered to be border plants and were not used for measurement. Field experiments were conducted at the experimental station of the Institute of Plant Sciences of the ETH in Eschikon near Zurich (47°26′ N, 8°40′ E, 550 m above sea level). The soil was an Eutric Cambisol (FAO classification) with a clay loam (CL) texture and a low content of organic matter (3%) (Richner *et al.*, 1996). Air temperature (thermistor YSI 400, Yellow Spring Instruments, Yellow Spring, OH, USA) and global radiation (BF2, Delta-T Devices, Cambridge, UK) were recorded at 15 min intervals, 2 m above the soil surface close to the experimental site.

Photosynthesis and chlorophyll fluorescence

To obtain light response curves from the parental lines, carbon exchange and chlorophyll *a* fluorescence were monitored with a portable photosynthesis system (LI-6400) equipped with a LI-6400–40 pulse-amplitude modulation fluorometer (Li-Cor, Lincoln, NE, USA). Light response curves were conducted at plants from the late sown set at the second leaf of seedlings in the 2nd leaf stage (11–12 June 2002) as well as during flowering at the second leaf above the ear (2–3 August 2002). The operating quantum yield of photosystem II photochemistry (ϕ_{PSII}) was calculated according to Genty *et al.* (1989). Other chlorophyll *a* fluorescence parameters were calculated using the 'lake' model according to Kramer *et al.* (2004). The fraction of *open* PSII reaction centres was estimated as

$q_L = ((F'_m - F')F'_o)/((F'_m - F'_o)F')$, the quantum efficiency of *open* PSII reaction centres as $\phi q_L = ((F'_m - F'_o)F')/(F'_m \times F'_o)$, the quantum efficiency of dissipation by down-regulation as $\phi_{NPQ} = 1 - \phi_{PSII} - 1/(F_m/F'_m + q_L(F_m/F_o - 1))$, and the quantum efficiency of other non-photochemical losses (non-light-induced, basal or dark, quenching processes) as $\phi_{NO} = 1 - \phi_{PSII} - \phi_{NPQ}$. The maximum fluorescence in the dark-adapted (F_m) and in the light-adapted (F'_m) state was determined by applying a 0.8 s saturating flash (~8300 μmol m^{-2} s^{-1}). To determine F'_o, a 3 s far red pulse was applied after the saturation pulse. For nomenclature of the fluorescence states see Rosenqvist and van Kooten (2003).

For QTL analysis in 2002, the operating quantum efficiency of PSII photochemistry (ϕ_{PSII}) of the $F_{2:3}$ population were recorded with the LI-6400. The light intensity was set at 400 μmol m^{-2} s^{-1}; the temperature of the sample chamber was 18 °C for plants sown early and 30 °C for plants sown late. These temperatures reflected the temperature in the field at the time of the measurements. Humidity and CO_2 concentration were the same as the ambient conditions. The chlorophyll *a* fluorescence was measured in the middle section of the third leaf after about 2 min adaptation to chamber conditions.

In 2003, the chlorophyll *a* fluorescence was recorded with a pulse-amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). The operating quantum efficiency of PSII photochemistry (ϕ_{PSII}) was measured from leaf discs; the discs had been punched from the middle of the third leaf and incubated, while floating on water, in a growth chamber for at least 30 min. The temperature of the growth chamber was 18 °C (early sowing date) and 25 °C (late sowing date). The light intensity was 400 μmol m^{-2} s^{-1}. The ϕ_{PSII} of six leaf discs of each experimental unit was measured. Furthermore, the maximum quantum efficiency of PSII primary photochemistry (F_v/F_m) of the third leaf was determined *in vivo* on the $F_{2:3}$ population in the field. F_v/F_m was measured by applying a 1 s saturation flash (>8000 μmol m^{-2} s^{-1}). The F_v/F_m measurements were conducted during the night from about 1 h after sunset until about 1 h before sunrise. In the plants sown early, F_v/F_m was measured from the evening of 16 May 2003 until the early morning of 18 May 2003. Measurements of the late-sown set were done from 6 June 2003 (evening) until 9 June 2003 (early morning). The F_v/F_m of 10 plants was determined for each experimental unit.

Morpho-physiological traits

The greenness of the third leaf was determined with a portable SPAD-502 chlorophyll meter (Minolta, Osaka, Japan). The measurements were performed on three parts of the middle section of the third leaf of six randomly selected plants per experimental unit.

The area of the third leaf was measured with a portable area meter, LI-3000A, (Li-Cor, Lincoln, NE, USA) equipped with a LI-3050A transparent conveyer belt. For each experimental unit, the leaf area of 10 plants was measured.

To determine shoot dry weight, 10 plants per experimental unit were dug out and cut at the coleoptilar node. The samples were washed and dried in an oven at 65 °C for at least 72 h. All the dried shoots of each line were pooled and ground prior to the determination of the nitrogen and carbon contents, which was performed with a Leco CHN-1000 elemental analyser (LECO, St Joseph, MI, USA).

In 2002, the morpho-physiological traits (leaf greenness, ϕ_{PSII}) were measured at the seedling stage on 29 and 30 May (early-sown plot) and 20 and 21 June (late-sown plot). In 2003, the measurements (leaf greenness, leaf area, shoot dry weight, and ϕ_{PSII}) were done from 19 to 21 May (early-sown plot), and between 16 and 18 June (late-sown plot).

Quantitative trait loci analysis

Means of the phenotypic traits of the $F_{2:3}$ were checked for normality of distribution as described by Shapiro and Wilk (1995) using the SAS PROC UNIVARIATE (SAS 8.2, SAS Institute, 1999–2001, Cary, NC, USA). The adjusted mean for QTL analysis was calculated by the Alpha lattice program (Alpha, CIMMYT, Mexico). All the QTL analyses for the individual environments were performed using QTL Cartographer version 1.17b (Basten *et al.*, 1994). The method of composite interval mapping (CIM), model 6 of the Zmapqtl program module, was deployed for mapping the QTLs and estimating their effects (Basten *et al.*, 2002). The genome was scanned at 2 cM intervals and the window size was set at 30 cM. Cofactors were chosen using the forward–backward method of step-wise regression at $p(F_{in}) = p(F_{out}) = 0.05$. A joint analysis of the phenotypic data for early and late sowing in both years, respectively, made it possible to evaluate the QTL-by-environment (Q×E) interaction (Jiang and Zeng, 1995). The presence of a QTL was declared significant when the likelihood of odds (LOD) value was higher than 3.50 for a single-trait analysis and higher than 4.43 for a joint analysis. These values correspond to a Type-I error rate (α level) of 0.021, assuming that all the chromosome arms segregate independently. An LOD threshold of 1.3 for a significant Q×E interaction was based on the Type-I error rate of a single locus for an F_2 with two degrees of freedom. Additive effects of the detected QTLs were also estimated by the Zmapqtl procedure of QTL Cartographer using hypothesis 31 (Basten *et al.*, 2002). The R^2 value (coefficient of determination) from this analysis indicated the percentage of phenotypic variance explained by marker genotypes at the locus.

Results

Environmental conditions

Figure 1 shows the daily mean temperature and daily global radiation throughout the experiments. In 2002, the average temperatures of 14 d before the harvest of the seedlings were 13.3 °C (early sowing) and 19.0 °C (late sowing). In 2003, the average temperatures were 13.9 °C (early sowing) and 22.0 °C (late sowing). Global radiation fluctuated throughout the seedling development, especially in the early-sown sets. The average global radiation of 14 d before the harvest of the seedlings were 18.6/18.4 MJ m^{-2} d^{-1} (2002/2003) for the early sowing and 24.2/27.3 MJ m^{-2} s^{-1} (2002/2003) for the late sowing.

Characterization of the parental lines

In the first year of the experiment (2002), the photosynthetic apparatus of the parental lines was studied by light response curves. The light response of photosynthesis showed that the photosynthetic capacity and efficiency of the chilling-sensitive genotype, ETH-DL3, were lower than those of the chilling-tolerant genotype, ETH-DH7, in young seedlings, which developed at low temperature (Fig. 2). Prior to measurement of the light response curves at the 2nd leaf stage, the average temperature of the 7 d before the measurements was 13.9 °C. The lower operating quantum efficiency of PSII (ϕ_{PSII}) in the sensitive compared with the tolerant genotype was mainly due to the lower quantum efficiency of *open* PSII reaction centres (ϕ_{qL}) and, to a lesser extent, to a decrease in the fraction of *open* PSII reaction centres (q_L). The difference in the efficiency of *open* PSII

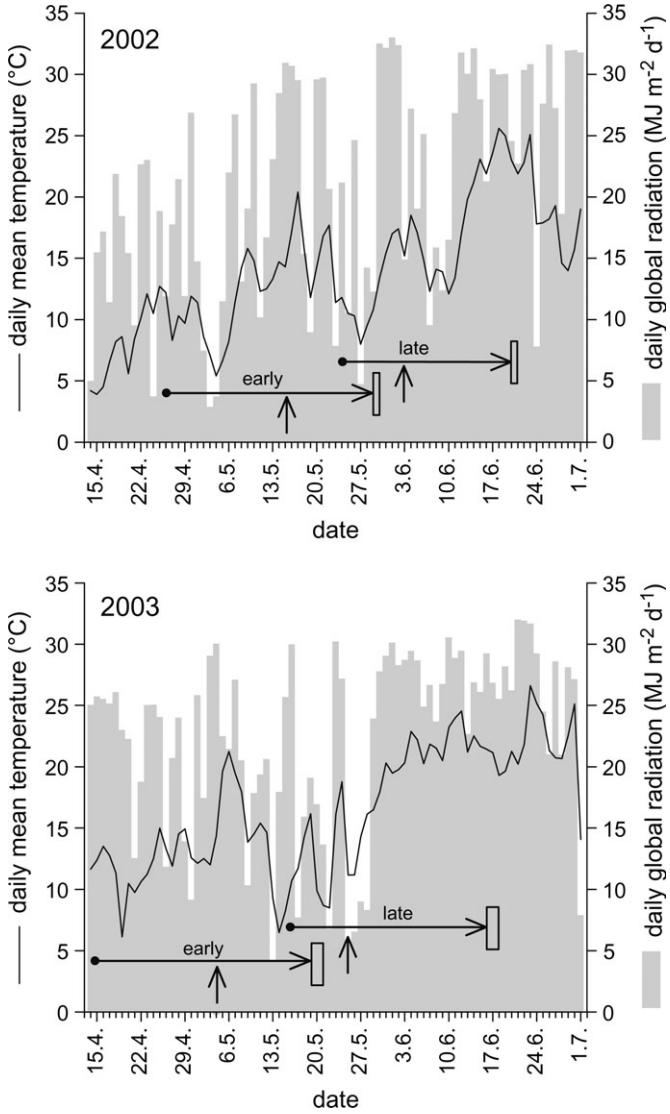

Fig. 1. Daily mean temperature and daily global radiation during the growing season in 2002 and 2003. The two sets of each year are marked by horizontal arrows. The dates of emergence are indicated by vertical arrows.

reaction centres seemed to be caused for the most part by non-light-induced quenching processes, as indicated by the higher value for ϕ_{NO} in the sensitive line, while the light response of the quantum efficiency for dissipation by down-regulation (ϕ_{NPQ}) revealed only a few differences between the two genotypes. The comparison with leaves developed at favourable temperature (2nd leaf above the ear during flowering) showed that differences between the two genotypes disappeared under optimal temperature conditions. Moreover, seedlings of the chilling-tolerant genotype, ETH-DH7, were characterized by a higher maximum quantum efficiency of PSII primary photochemistry (F_v/F_m) and a higher chlorophyll content; the latter was estimated from measurements of the leaf greenness (SPAD), especially of the early-sown sets (Table 1). The shoot dry weight and area of the third leaf was higher for the tolerant than for the sensitive genotype when plants were sown early. The opposite was observed when plants were sown late (Table 1).

Quantitative trait loci analysis

In the first experimental year, traits which characterize the photosynthetic apparatus and which can be measured in relatively short time, namely SPAD and ϕ_{PSII}, were determined in the segregating population. Interim conclusions, which were drawn after the first year and according to the results obtained under controlled conditions (Frachebound *et al.*, 2004), revealed additional traits of great interest for examination in the second experimental year.

In the $F_{2:3}$ population, the lines were significantly different for all the parameters at the early and late planting dates, with the exception of ϕ_{PSII} and F_v/F_m of the late planting date and F_m of both planting dates (Table 1). For the traits evaluated in both years, namely ϕ_{PSII} and SPAD, the combined data over environments was analysed to obtain the heritability. The heritability of ϕ_{PSII} was rather low (h^2=0.25) indicating that this trait was strongly affected by environment. For leaf greenness, a heritability of h^2=0.71 was determined.

For leaf greenness (SPAD) measured on the third leaf, six QTLs were detected from the seedlings sown early (Table 2). These QTLs were located on chromosomes 1 (181 cM), 2 (125 cM), 3 (102 cM), 4 (20 and 138 cM), and 10 (78 cM). An increase in leaf greenness was due to the alleles of the chilling-tolerant parent at most of these loci, except for the QTL at chromosome 3 and that at the beginning of chromosome 4. At the latter two loci, the greener leaves were due to the allelic contribution of the chilling-sensitive parent, ETH-DL3. The QTL for leaf greenness on chromosome 2 was not present in plants sown late. The joint analysis revealed that this QTL was stable across cold environments, indicated by the low Q×E interaction for the early sowing dates (Table 2). In plants sown late, four QTLs for leaf greenness were revealed. Three were also found in the plants sown early. Only the QTL at chromosome 9 (28 cM) was specific for the late-sown plants.

QTLs for ϕ_{PSII} were found mostly in plants sown early (Table 2). The most prominent QTL for ϕ_{PSII} was located on chromosome 6 at 225 cM; it was found in both years and explained 7.6% of the phenotypic variance of ϕ_{PSII} in 2002 and 20.4% in 2003. The increase in this trait was due to the allelic contribution of the chilling-tolerant parent. There were further QTLs for ϕ_{PSII} on chromosomes 2, 4, 8, and 9 detected in the early sown sets (Table 2). One of these QTLs, namely the one at chromosome 8, was also found in the late-sown set. In both sets it had a very low Q×E interaction indicating a high stability. The QTL at chromosome 9 seemed to be specific to the early-sown plants in

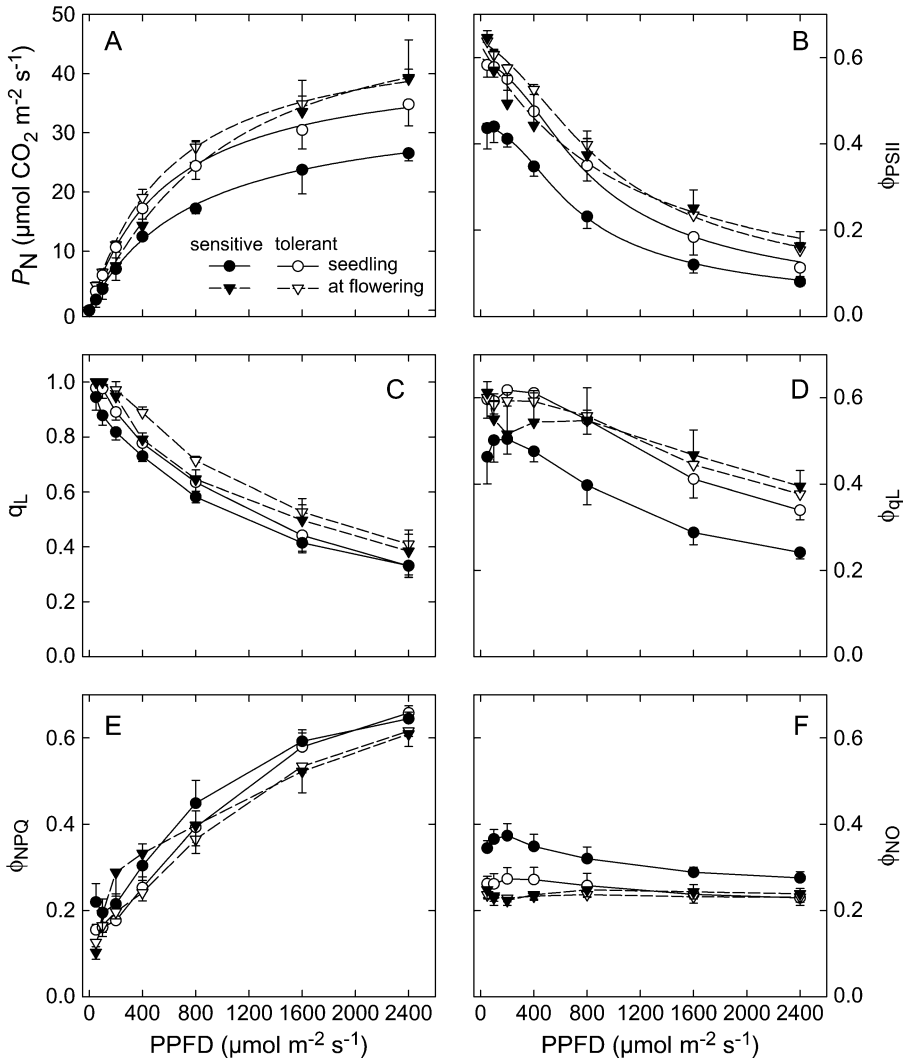

Fig. 2. Light response curves of photosynthesis (A), operating quantum efficiency of PSII (B), fraction of PSII centres in *open* states (C), quantum efficiency of *open* reaction centres (D), quantum efficiency for dissipation by downregulation (E), and quantum efficiency of other non-photochemical losses (F) in the chilling-tolerant (white symbols) and chilling-sensitive line (black symbols) at seedling stage (circles; 11–12 June 2002) and during flowering (triangles; 2–3 August 2002). Measurements were conducted at 20 °C at the second leaf of seedlings and at 25 °C at the second leaf above the ear during flowering. Values are means ±SD of four replications. The light response curves of photosynthesis and ϕ_{PSII} were fitted according to the model of Thornley (1976).

2002; here, it explained a large percentage of the phenotypic variance and was characterized by a high additivity.

Measurements of the maximum quantum efficiency of PSII primary photochemistry (F_v/F_m) were conducted in 2003. Two QTLs for F_v/F_m were common in both the early- and the late-sown sets; they were located on chromosome 2 (138 cM) and chromosome 6 (225, 219 cM) (Table 3). These two QTLs explained a high proportion of the phenotypic variance in the early and late-sown sets. However, their additivity was high only in the early-sown plants, while it was rather low in the late-sown set. Two additional QTLs were found for the plants of the early sowing date; they were located at the beginning of chromosomes 1 and 4. The additivity of these QTLs was negative, showing that the chilling-sensitive genotype carried the favourable allele for this trait. A decrease in F_v/F_m can be caused by an increase in F_o or a decrease in F_m. The QTL analysis revealed that the major QTL for F_v/F_m on chromosome 6 was also found for F_o but not for F_m. Since the additivity at this locus was positive for F_v/F_m and negative for F_o, the decrease in F_v/F_m seems to be due to an increase in F_o. By contrast, the QTL for F_v/F_m at chromosome 4 was found also for F_m, but not for F_o. Since the additivity was negative for F_v/F_m as well as for F_m, the decrease in F_v/F_m seemed to be caused by a decrease in F_m at this locus.

The QTL analysis revealed four QTLs for the area of the third leaf of the early-sown $F_{2:3}$ families and three QTLs for the late-sown set (Table 4). One QTL on chromosome 3 at about 100 cM seems to be common for early- and late-sown plants, although the peaks were 24 cM apart. Furthermore,

a second QTL at chromosome 3 (11 cM), one at chromosome 7 (95 cM), and one at chromosome 8 (74 cM) were found in the early-sown set. In the late-sown plants, a strong QTL was detected at chromosome 5 (29 cM). All the QTLs for leaf area were due to the allelic contribution of the chilling-tolerant parent, with the exception of the QTL at chromosome 1 (181 cM) identified in the late-grown set.

For shoot dry weight of the seedlings sown early, two QTLs were found on chromosome 5 (90 cM) and 8 (39 cM). The increase in shoot dry weight was due to the allelic contributions of the chilling-tolerant and the chilling-sensitive parent, respectively (Table 4). The QTL on chromosome 5 was also found in the late-sown set. In plants sown late, a second QTL was detected on chromosome 3 at 86 cM with negative additivity.

The QTL analysis of traits obtained from the element analysis showed that C:N ratio was mostly influenced by the nitrogen content (Table 4); in early-sown plants the two QTLs at chromosomes 1 and 6 and in the late-sown plants the QTL at chromosome 9 were common for nitrogen content and C:N ratio. While in the early-sown plants a high C:N ratio and, respectively, a low nitrogen content, was inherited by the chilling-tolerant parent, the situation for the QTL was the opposite for the late-sown set.

Table 1. *Means of leaf greenness (SPAD), operating quantum yield of photosystem II (ϕ_{PSII}), maximum quantum efficiency of PSII primary photochemistry (F_v/F_m), minimum fluorescence (F_o), maximum fluorescence (F_m), shoot dry weight (DW; g plant^{-1}), and area of the third leaf (LA; cm^2) of the parental lines (tolerant=ETH-DH7; sensitive=ETH-DL3) and the 226 $F_{2:3}$ families from their cross*

Trait	Env.[a]	Parental lines		$F_{2:3}$ population		
		Tolerant	Sensitive	Min	Max	Mean[a]
2002						
SPAD	E	33.0	21.3	17.4	34.8	24.6**
	L	45.5	37.3	34.2	47.7	40.8**
ϕ_{PSII}	E	0.33	0.28	0.21	0.39	0.31**
	L	0.52	0.51	0.45	0.56	0.52^{NS}
2003						
SPAD	E	37.4	19.0	17.2	38.0	29.2**
	L	46.8	38.3	39.1	50.4	44.6**
ϕ_{PSII}	E	0.42	0.27	0.20	0.41	0.32**
	L	0.37	0.32	0.40	0.57	0.48^{NS}
F_v/F_m	E	0.61	0.47	0.46	0.65	0.56**
	L	0.81	0.71	0.77	0.81	0.79^{NS}
F_o	E	0.31	0.33	0.24	0.41	0.30**
	L	0.26	0.24	0.20	0.29	0.24**
F_m	E	0.82	0.66	0.51	0.91	0.70^{NS}
	L	1.29	1.07	0.96	1.40	1.17^{NS}
DW	E	0.43	0.23	0.28	0.58	0.41**
	L	0.85	1.14	0.74	1.68	1.11**
LA	E	12.5	5.6	9.5	26.5	15.5**
	L	13.7	17.9	9.9	22.8	16.5**

[a] ^{NS}, not significant; **, significant at $P < 0.01$; Env., environment; E, early sowing; L, late sowing.

Discussion

The photosynthetic performance, but also the shoot biomass accumulation, of the two parental lines differed considerably when the plants were exposed to low temperature early in the growing season. This difference disappeared when the plants developed under favourable temperature conditions. The photosynthetic apparatus of seedlings grown under cold conditions in the field showed the typical symptoms of growth at suboptimal temperature,

Table 2. *Main characteristics of QTLs for leaf greenness (SPAD) and operating quantum efficiency of PSII (ϕ_{PSII}) of maize seedlings sown early or late with an LOD score above a threshold of 4.43 for the joint analysis*

Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; Joint, LOD score in the joint analysis of year 2002 and 2003; Q×E, LOD score value for QTL–environment interaction in the joint analysis of year 2002 and 2003; R^2, percentage of the phenotypic variance explained by genotype class at LOD peak; Add., Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).

Trait	Sowing	Chr	cM	Range	Nearest marker	LOD score				R^2		Add.	
						2002	2003	Joint	Q×E	2002	2003	2002	2003
SPAD	Early	1	181	149–197	mmc0041	4.18	5.31	8.22	1.65	4.4	4.2	1.00	1.29
		2	125	123–138	bnlg121	3.22	3.13	5.07	0.23	6.4	5.0	0.98	1.04
		3	102	82–122	mmc0022	6.66	1.33	7.01	2.59	10.6	1.7	−1.15	−0.80
		4	20	10–36	umc1276	0.15	4.90	5.11	2.39	0.0	7.9	−0.57	−0.93
		4	138	134–140	bnlg2291	0.18	4.54	4.78	2.36	0.1	7.0	0.51	0.93
		10	78	63–82	bnlg1712	0.77	4.70	4.80	0.91	1.0	4.2	0.66	0.89
	Late	1	165	157–189	umc1128	2.47	3.41	5.22	1.02	4.9	6.0	0.86	0.65
		3	110	72–134	mmc0022	4.04	8.82	11.29	1.58	7.3	10.3	−1.05	−1.22
		9	28	10–79	bnlg244	0.72	6.60	6.77	1.11	1.4	8.3	0.76	0.94
		10	92	39–122	umc1995	3.41	4.65	6.57	0.30	5.0	6.5	0.90	0.80
ϕ_{PSII}	Early	2	108	100–110	phi109642	4.06	0.49	4.67	0.78	9.3	0.5	0.011	0.008
		4	12	0–40	umc1276	1.07	6.29	6.98	1.98	0.9	8.3	−0.009	−0.013
		6	225	195–241	bnlg1740	5.88	17.98	21.34	5.75	7.6	20.4	0.014	0.021
		8	63	61–65	bnlg1782	1.91	3.12	4.59	0.41	3.5	2.3	0.010	0.009
		9	188	158–188	umc1277	5.59	2.82	9.24	1.37	24.8	3.6	0.020	0.022
	Late	8	66	65–66	bnlg1782	3.63	1.46	4.68	0.06	5.9	2.4	0.004	0.004

Table 3. *Main characteristics of QTLs with an LOD score >3.5 for photosynthetic maximum quantum efficiency of PSII primary photochemistry (F_v/F_m), minimum fluorescence (F_o), and maximum fluorescence (F_m) of the field experiment in 2003*

Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; R^2, percentage of the phenotypic variance explained by genotype class at LOD peak; Add., Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).

Trait	Chr	cM	Range	Nearest marker	LOD	R^2	Add.
Early sowing							
F_v/F_m	1	0	0–12	bnlg1112	3.74	4.1	−0.007
	2	138	98–154	dupssr21	6.79	12.5	0.017
	4	4	0–30	phi072	3.68	4.8	−0.008
	6	225	201–241	bnlg1740	18.22	23.8	0.023
F_o	6	227	195–241	bnlg1740	19.15	30.3	−0.023
F_m	4	6	0–38	phi072	4.52	8.2	−0.026
	8	65	32–86	bnlg1782	4.48	7.2	0.026
Late sowing							
F_v/F_m	2	138	97–154	dupssr21	4.02	9.7	0.003
	6	219	191–241	bnlg1740	15.55	30.4	0.005
F_o	6	205	176–225	bnlg1740	4.87	15.2	−0.006
F_m	–	–	–	–	–	–	–

Table 4. *Main characteristics of QTLs with an LOD score >3.5 for area of the third leaf (LA; cm^2), shoot dry weight (g $plant^{-1}$) carbon content (C%, g [C] g^{-1} shoot dry weight), nitrogen content (N%, g [N] g^{-1} shoot dry weight), and C:N ratio of the field experiment in 2003*; *see Table 3 for legends*

Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; R^2, percentage of the phenotypic variance explained by genotype class at LOD peak; Add., Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).

Trait	Chr	cM	Range	nearest marker	LOD	R^2	Add.
Early sowing							
LA	3	11	0–32	umc1394	4.23	10.6	0.015
	3	86	63–110	mmc0022	4.48	10.8	1.265
	7	95	66–108	umc1671	3.89	8.1	0.666
	8	74	55–88	mmc0181	4.52	7.6	0.602
Shoot DW	5	90	60–119	bnlg1118	6.55	12.8	0.028
	8	39	28–51	bnlg1863	6.63	15.2	−0.002
C%	–	–	–	–	–	–	–
N%	1	165	142–181	umc1128	3.88	7.7	−0.094
	6	235	189–241	umc1653	5.61	10.7	−0.103
C:N	1	167	142–185	umc1128	4.44	7.9	0.207
	6	235	199–241	umc1653	8.86	15.9	0.285
	8	66	57–74	bnlg1782	4.99	7.5	0.223
Late sowing							
LA	1	181	173–209	mmc0041	5.22	9.9	−1.362
	3	110	71–130	mmc0022	4.79	9.2	1.014
	5	29	7–66	umc1155	5.81	12.7	1.478
Shoot DW	3	86	53–116	mmc0022	4.80	11.3	−0.051
	5	92	68–111	bnlg1118	8.06	14.8	0.067
C%	3	210	186–225	bnlg1257	3.94	8.2	−0.201
N%	9	41	16–61	umc1033	4.09	8.1	0.163
C:N	9	41	14–61	umc1033	3.93	7.9	−0.399

namely low photosynthetic capacity and efficiency and low chlorophyll content, as demonstrated in several studies conducted under controlled environment conditions (Nie *et al.*, 1992; Haldimann *et al.*, 1996; Leipner *et al.*, 1997) and in the field (Andrews *et al.*, 1995; Leipner *et al.*, 1999). The regulation of the photosynthetic apparatus with respect to the fraction and the efficiency of *open* PSII reaction centres seemed to function similarly in both lines. However, seedlings of the chilling-sensitive line exposed to low growth temperature, were characterized by a higher yield for energy losses (ϕ_{NO}), which was not attributed to down-regulation; it indicated chilling-induced constitutive structural alterations of the photosynthetic apparatus.

The time of sowing and/or the climatic conditions during early seedling development also resulted in differential expression at several QTLs. Most of the identified QTLs derived from seedlings of the early-sown plots. Since the parental lines were selected for high or low operating quantum efficiency of PSII photochemistry (ϕ_{PSII}) at suboptimal growth temperature (Fracheboud *et al.*, 1999), this observation indicates that the selection method was efficient for the particular growth conditions. Moreover, the fact that the favourable alleles for most of the QTLs were inherited from the chilling-tolerant parent also shows the efficacy of the selection method.

Several genomic regions were found where different traits were under related genetic control. A major QTL for photosynthetic traits was located on chromosome 6 and was identified for the photosynthesis related parameters ϕ_{PSII}, F_v/F_m, and F_o in early-sown plants. This QTL was also found for F_v/F_m in the late-sown set, but with a considerably smaller additivity, indicating its greater importance under cool conditions. A previous study, conducted with the same plant material grown under controlled conditions, revealed a QTL at the same position and for the same traits in plants grown at 15 °C, but not in plants developed at 25 °C (Fracheboud *et al.*, 2004). Under controlled low-temperature conditions, there was a pleitropic effect between the photosynthetic traits and the shoot dry weight at this locus, which led to the assumption that photosynthesis limits dry matter accumulation at suboptimal growth temperature. Seemingly, this was not the case in the field. This might be due to the higher light intensity under natural conditions, since a decrease in the maximum photosynthetic efficiency has a relatively stronger effect on photosynthetic activity at low light intensity than it has at high light intensity. Moreover, the probability that growth is source limited is much larger under low light than under high light intensity conditions. Taken together, changes in photosynthetic efficiency will have a smaller effect on growth at higher light intensity, as in the field, than at lower light intensity as is usual under growth chamber conditions. Nevertheless, in early-sown plants in the field, a QTL for the C:N ratio was detected at this locus, indicating the involvement of the quantum efficiency of PSII in the overall carbon assimilation. Furthermore, the QTL analysis revealed a weak QTL

for shoot dry weight in the early-sown set at the end of chromosome 6 which was, however, with a LOD score of 1.9 below the threshold (data not shown). Since a QTL for leaf greenness was not detected at this locus, the molecular cause of the lower photosynthetic efficiency may be changes in the functioning of the photosynthetic apparatus or a feedback inhibition of photosynthesis, rather than a smaller amount of photosystems. This reduction in photosynthetic activity, however, seemed only partially to be the limiting factor for dry matter accumulation under field conditions. As discussed previously (Fracheboud *et al.*, 2004), an interesting candidate gene for this QTL might be *agp2* coding for the small subunit of leaf ADP glucose pyrophosphorylase (Table 5).

On chromosome 2 close to marker *dupssr21*, a common QTL was found for leaf greenness in early-sown seedlings and for F_v/F_m in early- and late-sown seedlings, the latter, however, with low additivity. Moreover, QTLs for leaf greenness, carbon exchange rate, and ϕ_{PSII} were identified at this position in this (Fracheboud *et al.*, 2004) and in another population (Fracheboud *et al.*, 2002) grown at suboptimal temperature under controlled conditions. The association between low chlorophyll content and reduction in the quantum efficiency of PSII might reflect a disturbance of the assembly of the photosynthetic apparatus, induced by low growth temperature. Aligning these results with the IBM2 Neighbor's consensus genetic map (Maize Genetics and Genomics Database, www.maizegdb.org) revealed the presence of an interesting candidate gene at this locus: *hcf106* (Table 5). The *hcf106* gene codes for the high chlorophyll fluorescence protein 106, which is a component of the ΔpH-dependent translocation pathway in the thylakoid membrane (for review see Mori and Cline, 2001). Nuclear mutation of *hcf106* results in a pale green, non-photosynthetic seedling, which releases absorbed light energy as chlorophyll fluorescence (Martienssen *et al.*, 1989), similar to plants developed at low temperature and carrying the allele at this locus from the chilling-sensitive line. The gene *hcf106c*, a homologue of *hcf106*, is located at chromosome 10, close to *umc1995*, the nearest marker of another QTL for leaf greenness identified in the present study (Table 5).

For leaf greenness, leaf area, and shoot dry weight a common QTL was located close to the centromere of chromosome 3. Similarly, several common QTLs for shoot dry weight and leaf area were found in maize seedlings grown in the greenhouse (Causse *et al.*, 1995). With respect to the QTL at chromosome 3, however, high shoot dry weight was related to a small leaf area. Since the relative growth rate (*RGR*) reflects the product of net assimilation rate (*NAR*) and leaf area ratio (*LAR*) (Evans, 1972), one would expect that a QTL for carbon assimilation rate would be present at this location, which exhibits the same direction of additivity as for shoot dry weight, thus counteracting the smaller leaf area. Beside the QTL for leaf greenness, a QTL for F_v/F_m with a LOD score of 2.4, which was, however, below the threshold, was identified close to the centromere of chromosome 3 in the late-sown seedlings (data not shown). Moreover, a QTL for carbon exchange rate, ϕ_{PSII}, F_v/F_m, and leaf greenness, with the favourable allele inherited from the chilling-sensitive line (ETH-DL3) as in the present study, was revealed at this position in seedlings grown at optimal temperature in growth cabinets (Fracheboud *et al.*, 2004), supporting the involvement of photosynthesis in this QTL. The complexity of this QTL makes an explanation of its mode of action difficult. It is possible that the gene or the genes behind this QTL are involved in the control of cell division and, therefore, affect the biomass accumulation and the photosynthetic activity by altering the morphology of the leaf. A nearby located potential candidate gene for this mode of action would be *myb2*, which codes the cell division control protein 5 (CDC5) (Table 5). Alternatively, changes in photosynthetic performance could be the primary cause,

Table 5. *Position of potential candidate genes in relation to the QTLs detected in this study and to common SSR markers from this population (ETH-population, ETH-DL3×ETH-DH7), from the IBM2 2004 Neighbor's map and the Pioneer composite 1999 map*

Marker/gene/QTL[a]	Position (cM)		
	ETH-population	IBM2	Pioneer
Chromosome 2			
bnlg1909	121	297	82
hcf106	–	309	80
[1]QTL SPAD	125 (123–138)	–	–
bnlg121	126	319	89
dupssr21	131	309	85
Chromosome 3			
bnlg1019	76	191	43
mmc0022	102	318	–
[2]QTL SPAD	110 (72–134)	–	–
myb2	–	347	78
sps2	–	358	82
bnlg1063	127	441	47
Chromosome 5			
bnlg1346	78	545	150
[3]QTL shoot DW	90 (60–119)	–	–
bnlg1118	93	590	149
nnr2	–	599	–
bnlg1695	123	657	149
Chromosome 6			
umc1859	187	391	–
bnlg1740	225	511	134
[4]QTL ϕ_{PSII}	225 (195–241)	–	–
umc1653	241	535	–
agp2	–	536	156
Chromosome 10			
bnlg1712	78	218	57
umc1995	84	246	–
hcf106c	–	246	–
[5]QTL SPAD	92 (32–122)	–	–
umc1930	104	307	–

[a] QTL position from [1], joint early; [2], joint late; [3], early 2003, [4], joint early; [5], joint late.

which consequently would affect shoot growth and leaf greenness and area. According to the IBM2 Neighbor's consensus genetic map, a potential candidate gene, which is located near the identified QTL and which may explain the pleiotropic effect at this locus by the latter mode of action is *sps2*, a gene for sucrose phosphate synthase (SPS) (Table 5). The effect of SPS activity on photosynthesis is well documented for C_3-plants overexpressing SPS (for a review see Huber and Huber, 1996). Moreover, an effect of SPS activity on chlorophyll content was observed in SPS transformants of *Arabidopsis thalina* (Strand *et al.*, 2003). However, its effect on photosynthesis in C_4-plants has not been extensively studied. It may be of less importance since a short-term feedback inhibition of SPS by sucrose seems not to occur in maize leaves (Lunn and Hatch, 1997). If the chlorophyll content is indirectly regulated by the activity of enzymes involved in carbon assimilation, as also found for Rubisco in the C_4-plant *Flaveria bidentis* (Kubien *et al.*, 2003), then one would expect that all major QTLs for photosynthetic activity are associated with QTLs for leaf greenness. Clearly, this was not the case regarding the QTL at the end of chromosome 6, indicating that changes in photosynthetic activity does not necessarily affect the chlorophyll content.

Only one QTL was identified for shoot dry weight which was stable in all the investigated environments. This QTL was located on chromosome 5 at about 90 cM, and the favourable allele was inherited from the chilling-tolerant parent. It was also detected for shoot dry weight in seedlings sown in the autumn which developed under cold conditions (data not shown). The QTL at chromosome 5 was not related to any other trait; and it was not identified under controlled conditions either for this trait or for any of the other traits (Fracheboud *et al.*, 2004). According to the IBM2 Neighbor's consensus genetic map, this QTL is located near the *nnr2* gene, whose product is NAD(P)H nitrate reductase (Table 5). There is a strong indication that nitrate reductase is important for this QTL as shown by Hirel *et al.* (2001) for another segregating population. In the latter population, a QTL for nitrate reductase activity and nitrate content was identified in the same region of chromosome 5 when the map was aligned to the IBM2 Neighbor's consensus map. Since this part of the chromosome has no influence on the C:N ratio, the gene or the gene cluster behind the QTL at chromosome 5 might influence the shoot dry weight through the availability of nitrogen for growth. On the other hand, if carbon limits growth, then a QTL for photosynthesis or leaf area would be expected at this locus, which was not the case. The hypothesis that the activity of nitrate reductase affects the shoot dry weight is also supported by the high correlation between nitrate reductase activity at the seedling stage and final plant biomass of European maize cultivars (Feil *et al.*, 1993).

Together with previous results obtained under controlled conditions (Fracheboud *et al.*, 2004), it is evident that the main QTLs involved in the functioning of the photosynthetic apparatus at low temperature are stable in cold environments. Furthermore, the QTL analysis indicated that the chilling-induced reduction in photosynthetic activity can be caused by perturbation in the assembling of the photosynthetic apparatus, as well as by reduced enzyme activity down-stream of the photosynthetic light reaction. The former was implied by the co-location of QTLs for photosynthetic efficiency and leaf greenness, the latter was indicated by QTLs which were found only for photosynthetic efficiency but not for leaf greenness. The functioning of the photosynthetic apparatus in plants developed at low temperature in the field, however, did not noticeably affect biomass accumulation, in contrast to the findings under controlled conditions. Nitrogen assimilation is probably more important for seedling growth in the field, because a larger leaf area, which may also positively influence growth, did not affect biomass accumulation of the investigated material either.

The comparison of the QTLs with QTLs of similar traits at the seedling stage in two other mapped populations (Fracheboud *et al.*, 2002; Causse *et al.*, 1995) revealed some agreement. Similarities between mapped populations were found when the populations were grown under similar conditions and when traits characterizing the physiology of the photosynthetic apparatus were considered (Fracheboud *et al.*, 2004), indicating that the genetic basis of the chilling-tolerance of photosynthesis is similar in different maize germplasms.

Supplementary data

The genetic map and the location of the QTLs for leaf greenness, ϕ_{PSII}, F_v/F_m, area of the third leaf, and shoot dry weight are shown on a supplementary figure available at JXB online.

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